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PRINCIPAL INVESTIGATOR: J. Wade Harper, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

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Houston, TX 77030

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The cell cycle is regulated by the action of a family of cyclin dependent kinases (Cdks) which catalyze particular cell cycle transitions. Cdks are positively regulated through interaction with cyclins and are negatively regulated through phosphorylation and through association with inhibitory proteins of the CIP/KIP and INK4 families. We initially identified p21CIP1 as an inhibitor of the G1/S kinase Cdk2. Since that time, we and others have identified additional p21CIP1 homologs including p27KIP1 and p57KIP2. Our research has focused on the role of p21CIP1 in development and cancer. We have found that p21CIP1 is expressed in a highly cell type specific manner during embryonic development and in adult tussues, being most highly expressed in terminally differentiated cells. Through analysis of p21 deficient mice, however, we have found that p21CIP1 is not required for normal development but fibroblast from p21 deficient mice have a defect in the G1 DNA damage checkpoint regulated by p53, indicating that tumor suppression by p53 is complex. Current studies are aimed at understanding in greater detail the function of p21 and its possible role in human cancers.

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Introduction

Proliferation of eukaryotic cells is regulated primarily at two points in the cell cycle, in G1 prior to entry into S-phase and in G2 prior to entry into mitosis. The decision of whether to commit to a round of cell division or exit the cell cycle is made at a point in G1, referred to as the restriction point in mammalian cells[1-4] or START in yeast[5]. In fibroblasts, passage through the restriction point depends critically on the signals that are received through mitogen activated pathways, but once this point is passed, cells are committed to S-phase and the remainder of the cycle in a mitogen independent manner[4]. In somatic tissues, passage through the restriction point is thought to be the primary event controlling cell proliferation. Therefore, elucidating how positively and negatively acting genes function to regulate the G1/S transition and how mutations in these genes disrupt normal cell cycle control has been a primary focus of cancer research. Central to this focus has been the investigation of the role of cyclin-dependent kinases (Cdk) in the control of cell proliferation.

Cyclins, Cdks, and positive growth control. Cdks are protein kinases that require association with cyclins and phosphorylation for activity[5-7]. Currently, there are seven *bone fide* Cdks and several others related by sequence[8]. Biochemical and genetic data from several systems have demonstrated that cyclins promote cell cycle transitions via their ability to associate with and activate their cognate Cdks[5-12]. D-type and E-type cyclins function in the G1 phase of the cell cycle[6, 10, 13-16], and overexpression of cyclin D1 or cyclin E shortens G1 and accelerates entry into S-phase[1, 3, 17, 18]. A large body of correlative evidence exists linking deregulated cyclin expression to cancer. Amplification of cyclins, D1, D2 and E have been identified in several tumors[19-23]. Cyclin D1 was identified as the PRAD1 oncogene [24]. Cyclin A was identified as the site of integration of HBV in a hepatocellular carcinoma[25]. Taken together, these observations suggest that inappropriate activation of Cdks is a mechanism that cells frequently use to reach the oncogenic state.

D-type cyclins associate with Cdk4 and Cdk6 kinases and can phosphorylate and inactivate Rb[6, 26-29]. Since D-type cyclins are required for proliferation of tissue culture cells only if they have an intact Rb gene, it is thought that Rb inactivation is their primary role, however this has not been rigorously demonstrated in animals. Cyclin E binds to and activates Cdk2 and considerable evidence has accumulated indicating that cyclin E/Cdk2 is the primary kinase involved in the G1/S transition[14, 15, 30-33]. In addition, a close homolog of Cdk2 - Cdk3 - is also thought to play a unique role in the G1/S transition[31]. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition [34-36], while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry.

Since the controls utilized during development to regulate cell proliferation are similar to those utilized in maintenance of the non-proliferative state in differentiated tissues, it is likely that these controls are reactivated or overcome in cancer. A primary example of this comes from our observation that cyclin D1 is expressed at extremely high levels in the retina and is required for its development [37], as demonstrated by the fact that mice lacking cyclin D1 have a major defect in retinal cell proliferation during development. Presumably the inability to properly develop the retina in cyclin D1 mutants reflects an inability to overcome Rb. In what is clearly more than a coincidence, the retina is the same tissue in which high frequency tumors arise in Rb mutant humans. It is therefore likely that the Rb protein is important in both development of that tissue and its maintenance in the non-proliferative state. Our understanding of the links between development and cancer is in its infancy and is an area in which there is a great need to increase our knowledge base.

Tumor suppressor proteins and negative growth control. Much of our knowledge about negative regulation of cell cycle entry emanates from studies of two tumor suppressor genes, Rb and p53[29, 38]. Mutations in these are found frequently in diverse types of human cancers[39,40],

and reintroduction of wild-type genes into p53⁻ or Rb⁻ tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis(28, 41, 42).

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors(39) including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included(43). p53 deficient mice are prone to the spontaneous development of a variety of tumor types(44). Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53(45-53). p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification(54, 55).

Much is known about the function of Rb in cell cycle control and the large number of proteins with which it interacts (29). The current view is that hypo-phosphorylated Rb functions during G1 in part to block the activity of E2F and related transcription factors that are required for the expression of genes involved in S-phase. Hyper-phosphorylation of Rb or association with DNA tumor virus oncoproteins such as E1A results in release of E2F and is correlated with passage into S-phase.

These data are consistent with a model in which increased cyclin/Cdk activity in tumors, whether by increased cyclin expression or decreased negative regulation, can overcome the cell cycle repression function of Rb via direct phosphorylation and inactivation of its growth inhibitory function. Rb therefore acts as a potential energy barrier in the pathway that cyclin/Cdks must overcome to activate cell cycle entry. Removal of the barrier (Rb) may reduce the levels of kinase activity required, but some Cdk kinase activity is still required for the process of DNA replication and can therefore act as a target of further negative regulation. In this model, p53 acts to reduce the frequency of mutations that lead to altered growth control and to kill cells that have undergone extensive damage or are inappropriately growing. To fully understand this aspect of cancer, cell cycle dysfunction, it is imperative that we have a complete understanding of the regulation of cyclin dependent kinases and their regulators in the tissues of interest.

Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control. Cdk inhibitory proteins are a group of proteins that associate with and inhibit Cdks. These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life. At the time of submission of this grant in December 1993, the first mammalian Cdk inhibitors p21$^{CIP1/WAF1}$ (57-600) and p16^{INK4a} (61) had only recently been identified. Subsequently, we and others identified additional inhibitors including p27, p57, p15, p18, and p19 (refs 61-65, 77, 78, 81, 82). We identified p21^{CIP1} in a two-hybrid screen designed to identify proteins that associate with Cdk2 (57). Importantly, this protein was simultaneously cloned by several other laboratories. p21 was cloned as a p53 activated gene by the Vogelstein laboratory (59), as a Cdk associated protein by the Beach laboratory (58), and as an S-phase inhibitory cDNA in senescent cells (60). At that time there were many unanswered questions regarding the role p21 in cell cycle control and cancer. What was known was that p21 could bind and inhibit a number of Cdks, that it was transcriptionally regulated by p53 overexpression, and that its chromosomal location did not mark it as an obvious tumor suppressor. In addition, it was not known whether p53 is the only regulator of p21 expression, how p21 might be used during development, or whether loss of p21 expression contributes to cancer.

Much more is now known about these inhibitors, their association with Cdks and other molecules, their potential roles in development , how they are regulated, and their potential roles as tumor suppressors (79,80). Some of what is known about these inhibitors is summarized in Table 1.

The goals of our work were: 1) to determine the pattern of expression of p21 during embryogenesis and in adult tissues using in situ hybridization, 2) to construct mice lacking p21, 3) to analyze the phenotype of mice lacking p21, 4) to examine the status of p21 in breast tumors and tumor cell lines, and 5) to characterize the regulation of p21 in the cell cycle and to identify interacting proteins. To date, we have made significant progress on these initial goals. Our progress in these areas is summarized below.

Body

Aim 1: Expression of p21 during development and in adult tissues

The goal of this aim was to examine whether expression of p21 is regulated during development and in adult tissues, to determine if there is any cell type dependence on expression, and to determine whether p21 expression is dependent upon the transcription factor p53. While p21 expression after DNA damage is dependent upon p53, it was not clear whether normal expression required this transcription factor.

To address this question, we examined expression of p21 during mouse development using primarily the approach of in situ hybridization. Much of the results have been published(72) and will be summarized here. We found that p21 expression during embryonic development is extremely cell type dependent and occurs primarily in cells that are thought to be terminally differentiated, correlating with cell cycle arrest. The most striking expression pattern occurs in ht dermomyotome where p21 induction is paralleled by that of myogenin. Myogenin is a basic HLH transcription factor which functions in the muscle cell differentiation pathway and is expressed in cells that have already committed to the muscle cell differentiation program and are arrested. In addition to these sites of expression, p21 is also expressed in the respiratory epithelium, in embryonic skin, cartilage, hair follicles, and the apical ectodermal ridge of the limb bud. All of these tissues are thought to be terminally differentiated at the time of p21 expression. In adult tissue analyzed by insitu hybridization, we found mixed patterns of expression. In some tissues such as liver and skeletal muscle, expression appeared to be homogeneous while with other tissues such as stomach and intestine, selective expression of p21 was observed in the columnar epithelium. Consistent with the expression patterns observed, we and others have found that the levels of p21 are increased in a number of in vitro cell differentiation systems. Our studies have focused on muscle cell differentiation where we found that p21 levels increase during in vitro differentiation and myotube formation. (72) Similar results were obtained independently by Dr. A. Lassar's laboratory (74,75). Although transfection of MyoD expression plasmids leads to induction of p21 expression, mice lacking MyoD express p21 at normal levels suggesting that other redundant pathways are operating.(72)

To address whether p21 expression during development depends on p53, we performed in situ hybridization on mice lacking the p53 gene.(72) We found that basal levels of p21 are not altered in these mice, indicating that p53 does not normally regulate the basal expression of p21. Similar results were obtained by jacks and coworkers. Currently, little is known about the transcription factors that regulate p21 expression but it has recently been shown that the STAT family of transcription factors can bind the p21 promoter and activate transcription.

To further investigate the potential involvement of p21 in breast development, we have looked at p21 expression in virgin mice, in pregnant mice undergoing massive epithelial proliferation, and in lactating breast. As shown in Figure 1, p21 is essentially absent from virgin and pregnant epithelial ducts but mRNA levels increase substantially in lactating glands. This suggests a role for p21 in proliferative arrest concomitant with lactation. Aspects of these studies are addressed further under specific aim 3.

In our statement of work, we anticipated that these expression experiments would be completed by the end of year 2. For the most part, we consider this aim essentially completed as proposed.

Aims 2 and 3: Construction and analysis of mice lacking p21.

As detailed in last years annual report, we have collaborated with Chuxia Deng and Phil Leder in the analysis of mice lacking p21. Using standard gene targeted procedures, exon 2 of p21 was deleted in ES cells and chimeric mice transmitting the mutation were bred to

homozygosity.[56] We found that p21-/- mice display no obvious developmental phenotype. Mice are born at the expected Medelian ratios and are of normal size. $p21^{-/-}$ mice were monitored for evidence of illness or tumor formation weekly up to 7 months and peripheral blood examinations, which showed no evidence of red or white blood cell abnormalities, were carried out at 4 months of age. Histological sections from several organs, such as muscle, testis, vertebral bones, brain, were examined and were found to be normal. These results indicate that p21 is not required for normal development in the mouse. It is possible that other CKIs can substitute for loss of p21 during differentiation and other processes that require cell cycle arrest through CKIs. Since mice lacking other CKIs has either been generated (p27 and p16) or are in the process of being generated (see below), this possibility can potentially be addressed through the construction of multiply mutant mice.

Based on the fact that p53 null mice are viable but develop tumors at an accelerated rate, we thought it possible that p21-/- mice would perhaps be cancer prone. However, mice have now been in existence for greater than 2 years with no obvious increase in tumor frequency noted. These data indicate that p21 is not a tumor suppressor in the classical sense but the possibility still exist that it's loss can contribute to transformation in combination with other mutations.

While loss of p21 does not affect viability or tumorigenesis in the mouse, there existed the possibility that loss of p21 affected the p53-dependent checkpoint pathway operating in G1 in response to DNA damage. To examine this question, we established mouse embryonic fibroblasts (MEFs) from day 14 embryos of the three possible genotypes, wild type, $p21^{+/-}$ and $p21^{-/-}$. and examined whether these cells arrested in G1 in response to various kinds of DNA damage. Previous studies have shown that p53-/- MEFs are fully defective for G1 arrest in response to γ-irradiation (IR) and in addition, p53-/- thymocytes do not undergo p53-dependent apoptosis in response to IR. Using bivariate fluorescence activated cell sorting procedures, we found that p21-/- fibroblasts were defective in G1 checkpoint function, although the defect was not as severe as the defect found in p53-/- fibroblasts. In wild-type cells, 50% of the cells arrest in response to IR while only 2% arrest in the case of p53 deficiency. When p21 is absent, an intermediate level of 20% arrest. This result indicates that p21 is required for the full response to p53 activation but that additional pathways may exist that regulate G1 arrest independent of p21. Similar results were obtained with the nucleotide pool perturbation agent PALA but in this case, the severity of loss of p21 was essentially identical to that of p53 deficiency.

In contrast with the results from these two checkpoint assays, we found that thymocytic apoptosis and the G2 checkpoint in response to spindle malfunction are intact in p21-/- cells. Taken together, these results indicate that p21 action in response to p53 function may be limited to the G1 checkpoint function (see diagram in Figure 2). In addition, this G1 checkpoint pathway has additional components which have yet to be identified.

Completion of task 2 has occurred on schedule and further analysis of these animals in task 3 is continuing.

Aim 4: Analysis of p53 and CIP1 in human breast tumors

A number of laboratories have examined a large number of multiple tumor types, including breast tumors, for mutations in the p21 gene. With the exception of prostate where 2 inactivating mutations have been identified, there is a scarcity of mutations in the p21 gene. This indicates that p21 is not a critical mutational target in breast but does not exclude the possibility that its activity and or abundance is not affected by oncogenic mechanisms. This being the case, it is important to determine whether p21 expression is altered in breast cancers using immunohistochemical approaches. In collaboration with K. Keyomarsi, we have found that p21 is expressed in many normal mammary epithelial cell lines but is absent from most transformed mammary epithelial lines examined. While correlative, it is possible that loss of p21 expression is unrelated to transformation. To examine this question in greater detail, we have recently initiated a collaboration initially through E. Liu with the University of North Carolina SPORE for breast cancer to look at the expression of p21 in normal breast tissues and breast cancer samples available through the SPORE. These studies are in the early stages and no data is available at present.

However, in order to facilitate these studies we have collaborated with E. Harlow to generate a panel of monoclonal antibodies against p21 which can be used for immunohistochemistry on tissue sections. A manuscript describing the characteristics of these antibodies is currently in press (87). Briefly, we have identified a number of antibodies which work well both for immunohistochemistry, immunoblotting and immunoprecipitation. In a parallel line of study funded through the Baylor SPORE in prostate cancer, we have found using these antibodies that p21 is normally expressed at high levels in the epithelium of normal prostate glands but levels are greatly reduced or absent in ~70% of all prostate cancers examined (unpublished data). We plan to examine normal breast tissues to determine the normal pattern of p21 expression and to look for p21 expression in tumor sections, including invasive carcinoma and DCIS. These studies are anticipated to continue through the third year of the grant as indicated in the statement of work.

Aim 5: Analysis of p21 and associated proteins

The goal of this aim was to study the mechanism of action of p21, to identity association proteins, and to develop a genetic screen in yeast that would allow proteins capable of disrupting the p21/Cdk2 interaction to be cloned. We have made significant progress toward the first of these goals. The third sub-aim was attempted but we have thus far been able to express high enough levels of p21 to block proliferation of yeast. These experiments are not being pursued at present. As detailed in the previous progress report, we have found that p21 associates directly with PCNA [(67)]. Similar results have been reported by others.[(68, 85)] In addition, we have characterized the mechanisms of inhibition of Cdks by p21 in detail, looking both at inhibitory constants as well as associations in vitro and in vivo.[(66)] These data suggest that p21/kinase complexes can contain substantial kinase activity when measured in vitro in a conventional I.P. format. The relevance of this observation to in vivo function is unclear at present, but the phenomena has been observed by others.[(84)]

During the past year, we have performed an additional set of experiments aimed at determining whether other Cdk inhibitors might exist in mammary tissue. The impetus for this comes from the findings in specific aim 1 where we found that p21 expression is highly cell type specific. In addition, preliminary analysis of p18, p19, p27, and p57 indicate that they too also display a high degree of cell type specificity in there expression patterns. This being the case, it is conceivable that particular cell lineages have CKIs and other Cdk regulators that have yet to be identified. Essentially all of the CKIs identified to date are derived from cDNA libraries made from tissue culture cells many of which are transformed. Such cells may have lost expression of particular CKIs due to mutation or may have never expressed a particular CKI to begin with.

As part of an effort to identify new cell cycle regulators in important cancer prone tissues, we constructed new two hybrid libraries using mRNA derived from either human mammary tissue, mouse mammary epithelium, and human prostate. These libraries contain $>10^7$ individual recombinants with average insert sizes of 1.5 kb. This complexity is much larger than most of the available libraries. In addition, we have engineered new antibody epitopes into the original two hybrid vector to facilitate analysis of the protein identified.

Currently, we have performed two screens with each of these libraries, one searching for Cdk2 interacting proteins and one searching for Cdk6 interacting proteins. Funding for screening of the prostate library is from another source but is included here for comparison with the breast libraries. The results are summarized in Table II.

In every case, most of the genes identified were already known Cdk binding proteins, including CKIs, cyclins and Ckshs proteins. With the human breast library and Cdk2 as the interaction target, we identified primarily p21 (79%). Six additional novel cDNA were cloned and are currently in the early stages of analysis. Interestingly, the mouse library gave a substantially different distribution of cDNAs. In particular, Ckshs2, a Cdk binding protein whose function is not completely clear, was the most abundant cDNA followed by p21. Five novel cDNAs were also identified in this screen. It is currently unclear whether the differences in distribution found in the mouse and human breast libraries simply reflect different cell populations in the RNA used for

library constructions or whether expression of genes in mouse and human are different. The source of human RNA was reductive mammoplasty while the RNA for the mouse library was derived largely from virgin epithelial ducts. It is possible that the cells used for human RNA preparation contained a substantial fraction of fat cells which would bias the cDNA distribution.

In the Cdk6 screen, we also found major differences in the cDNAs obtained with the human and mouse libraries. As expected, most of the clones were p16 family members. We did not expect to find p21 family members in this screen since p21 association appears to be greatly enhanced by cyclin binding and Cdk6 does not effectively interact with yeast cyclins. From these screens, we identified 4 novel cDNAs which are currently being characterized. We currently do not know whether any of the novel genes are inhibitors or interact with Cdks in tissue culture cells.

Conclusion:

The last two years has seen a virtual explosion in our understanding of the mechanisms regulating cell cycle progression. Much of this concerns the role of Cdk inhibitors in cell cycle control. Our work funded under this grant involves an analysis of the role of p21 in mammary and potentially other cancers. Our approach has been to address patterns of expression during development, to determine whether p21 is required for the p53 checkpoint, tumor suppression, and or development through analysis of mice deficient in p21, and to understand mechanistic aspects of p21 function within the context of other CKIs including p57. Our major contribution has been to demonstrate that p21 is not required for the tumor suppression function of p53 at least in the mouse but that it is involved in G1 checkpoint control. This is an important finding since it focuses mechanistic studies on p53 to other pathways which may be important for tumor suppression such as apoptosis.

During the coming years, we plan to continue our analysis of p21 deficiency. We are currently in the process of generating mice lacking the p57 gene. Once these mice are available (assuming they are viable), we will mate these mice with p21 deficient mice to look for collaborative phenotypes. Other studies ongoing in other laboratories are seeking to generate p21-/-:p27-/- mice. Ultimately, through analysis of multiply mutant animals, we may begin to understand what activities are unique to individual CKIs and which are redundant. In addition, we will continue our analysis of p21 expression in normal breast tissues and tumors primarily using immunohistochemical approaches. In addition, we will continue our analysis of Cdk2 and Cdk6 interacting cDNAs we have identified using mouse and human breast cDNA libraries. In particular, we will test whether the encoded cDNAs associate with Cdks in cells, whether the function as inhibitors or activators of Cdks, and whether they are involved in cell cycle control.

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Appendix

Tables 1-2.

Figures 1-2.

Figure Legends

Figure 1. Expression of CIP1, KIP2, and D-type cyclins in devloping mammary glands. In situ hybridization was performed on virgin, pregnant, or lactating mammary glands using established techniques. mRNA signal - Red, nuclei signal - blue.

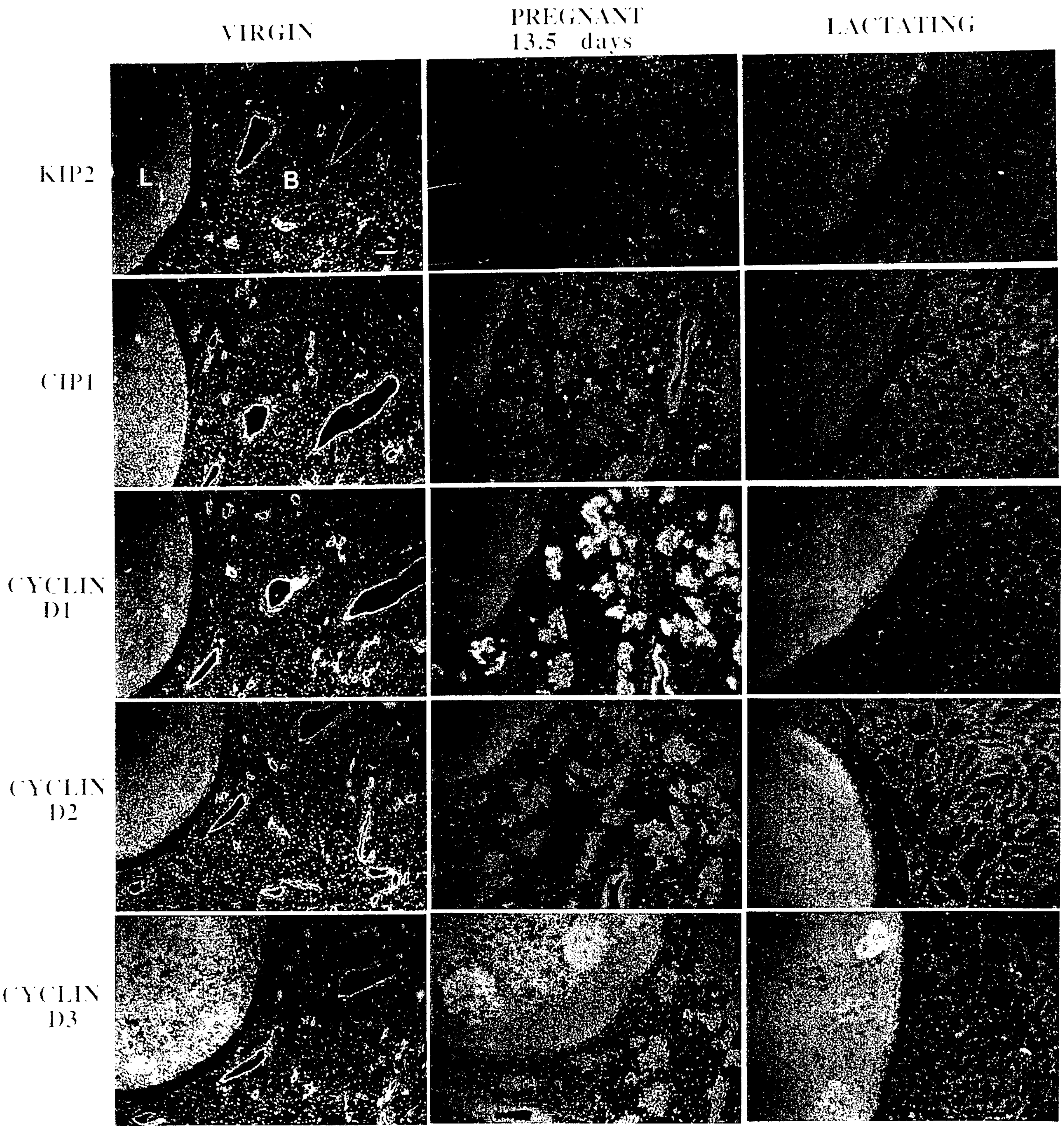

Figure 2. A Current View of the Integrated Actions of p21 and p53

DNA damage leads to stabilization and activation of p53, possibly via the ATM gene product. The activated p53 protein (p53*) induces transcription of $p21^{CIP1/WAF1}$ and other genes involved in cell cycle arrest and DNA repair. Under certain conditions that are not completely understood, p53 activation can lead to apoptosis. In response to γ irradiation, p53-dependent cell cycle arrest in G1 functions by both $p21^{CIP1/WAF1}$-dependent and independent mechanisms that are only partially redundant, while p53-dependent apoptosis in response to γ irradiation does not require $p21^{CIP1/WAF1}$. In mouse cells, p53 also regulates re-entry into S-phase when mitosis is blocked, as in the case of the anti-microtubule agents colcemid and nocodazole (54). This function is independent of $p21^{CIP1/WAF1}$. p53 may also regulate ploidy in the absence of spindle interference because untreated $p53^{-/-}$ cells rapidly increase ploidy with increased passage.

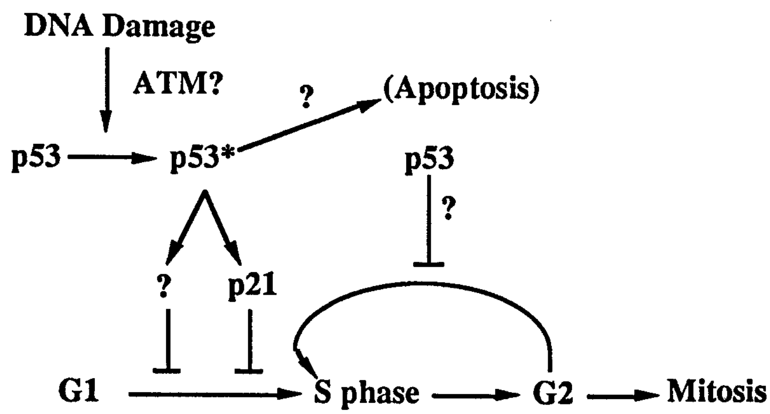

Table 1. Mammalian Cdk Inhibitors

Inhibitor	Primary Target	Chromosomal Location	regulator(s)	Comments
Ankyrin Family				
$p15^{INK4b}$	Cdk4,6	9p21	TGFβ	frequently deleted in glioblastoma
$p16^{INK4a}$	Cdk4,6	9p21	?	tumor suppressor (melanoma)
$p18^{INK4c}$	Cdk4,6	1p32	?	not yet associated with cancer
$p19^{INK4d}$	Cdk4,6	19p13	?	not yet associated with cancer
Dual-Specificity Family				
$p21^{CIP1/WAF1}$	Cdk2,3,4,6	6p21	p53,TGFβ MyoD	functions in G1 checkpoint
$p27^{KIP1}$	Cdk2,4,6	12p12-13	cAMP Rapamycin	not yet associated with cancer
$p57^{KIP2}$	Cdk2,3,4	11p15.5	?	candidate tumor suppressor for both sporadic and familial cancers

Table II. Summary of Cdk2 and Cdk6 interacting clones identified in mammary cDNA libraries

probe	library	cDNA	number of clones recovered	%
Cdk6	m. breast	p19	22	25
		p18	16	18
		p16	0	0
		p15	11	13
		cyclin D1	4	5
		cyclin D2	8	10
		cyclin D3	2	2.5
		novel MB1	23	25
Cdk6	m. breast	p19	3	4
		p18	11	16
		p16	35	50
		p15	17	23
		cyclin D1	1	1
		cyclin D2	1	1
		cyclin D3	0	1
		novel HB1	3	3
Cdk6	prostate	p19	26	43
		p18	11	18
		p16	1	1
		p15	0	0
		cyclin D1	1	1
		cyclin D2	2	2
		cyclin D3	3	3
		cyclin I	1	1
		novel HP2/HB1	4	4
		novel HP4	2	2
		novel HP6	1	1
		novel HP7	1	1

Table 2. Cont.

Cdk2	h. breast	p21	46	79
		novel HB2	4	8
		other independent novel cDNAs	5	9
Cdk2	m. breast	p21	9	21
		p27	1	2
		Ckshs1	4	10
		Ckshs2	13	31
		ubiquitin	2	4
		γ-actin	1	2
		Gα	1	2
		other independent novel cDNAs	5	9

<u>Personnel</u>
J. Wade Harper
Jeff Winston
Chang Bai
Lilia Stepanova
Xiaohong Leng
Stephen J. Elledge

Publications

Flores-Rozas, H., Kelman, Z., Dean, F., Pan, Z-Q., Harper, J.W., Elledge, S.J., O'Donnell, M., Hurwitz, J. (1994). Cdk-interacting protein 1 directly binds with PCNA and inhibits replication catalyzed by the DNA polymerase δ holoenzyme. *Proc. Natl. Acad. Sci. USA* 91, 8655-8659.

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